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Award Number: DAMD17-00-1-0248

TITLE: Pharmacogenetic Factors Contributing to Variation in Response to Tamoxifen at Raloxifene

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REPORT DATE: July 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2004	3. REPORT TYPE AND DATES COVERED Final (1 Jul 2000 – 30 Jun 2004)

4. TITLE AND SUBTITLE
Pharmacogenetic Factors Contributing to Variation in Response to Tamoxifen at Raloxifene

5. FUNDING NUMBERS
DAMD17-00-1-0248

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of these studies was to elucidate the pharmacogenetic factors that contribute to variation in human response to tamoxifen (TAM) and raloxifene (RAL). We had previously identified and partially characterized common genetic polymorphisms in two human drug -metabolizing genes, SULT1A1 and UGT1A6. We hypothesized that these polymorphisms contributed to variation in TAM or RAL metabolism. These studies were divided into three aims with the purpose of 1) biochemically characterizing the contribution of these enzymes to the metabolism of TAM and RAL; 2) developing cell model systems to study allele-specific differences in cellular response to these molecules and; 3) perform a clinical pharmacogenetic study to evaluate the association of common genetic polymorphisms in drug metabolizing genes with variable clinical response to TAM. We determined that SULT1A1 and UGT1A6 contributed to the inactivation of 4-hydroxytamoxifen (OHT), the active metabolite of TAM, and that a separate enzyme, UGT1A9 catalyzed the glucuronidation of RAL. We established MCF-7 breast cancer cell lines stably expressing the wildtype and variant SULT1A1 alleles and have measured allele-specific differences in the response of these cells to estrogens and OHT. These studies suggest that pharmacogenetic factors might contribute to variable cellular response to antiestrogens.

14. SUBJECT TERMS
SULT1A1, UGT1A6, pharmacogenetics, tamoxifen, sulfation and glucuronidation

15. NUMBER OF PAGES
51

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Rebecca Blanchard Raftogianis

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INTRODUCTION

The goals of this proposal are to elucidate the pharmacogenetic factors that influence cellular response to tamoxifen (TAM) and raloxifene (RAL). Specifically, the work described represents a stepwise approach to the study of genetic polymorphisms in human sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) genes. Those studies will progress from basic biochemical studies to the use of cell models and will culminate in a clinical pharmacogenetic study. In the first aim, we proposed to biochemically characterize the capacity of wildtype and variant SULT1A1, SULT1A2 and UGT1A6 proteins to conjugate 4-hydroxytamoxifen (OHT) and raloxifene RAL. The second aim focussed on the development of cell models to study allele-specific differences in cellular response to these antiestrogens. Finally, the third aim will determine the association of genetic polymorphisms in several metabolic pathways with human response to TAM in a clinical setting.

BODY

Specific Aim1. Biochemically characterize the capacity of recombinant wildtype and variant SULT1A1 and UGT1A6 proteins to conjugate OHT and RAL in *in vitro* assays. We proposed four sub aims associated with this aim—each to be completed within the first year of funding. Those included:

1) Generation of recombinant wildtype and variant UGT1A6 allozymes
2) Biochemical characterization of the SULT1A1 allozymes with regard to their capacity to sulfate OHT and RAL.
3) Optimization of the glucuronidation assay.
4) Biochemical characterization of the UGT1A6 allozymes with regard to their capacity to glucuronidate OHT and RAL.

In our previous progress reports we presented data regarding identification of novel UGT1A6 polymorphisms and biochemical characterization of SULT1A1 and UGT1A6 allozymes with OHT as a substrate. We also reported on the association between UGT1A6 alleles and level of enzyme activity in a bank of human liver tissues as well as differences in the biochemistry of UGT1A6 alleles toward several substrates. Those data suggested that the UGT1A6*2 allele, when expressed homozygously was significantly more active than wildtype (UGT1A6*1). A notable finding was that when UGT1A6*2 was expressed heter0zygously with UGT1A6*1, tissues exhibited significantly lower activity than wildtype or *2 expressed homozygously (Nagar et al., *Pharmacogenetics*, in press). This phenotype departed from the expected co-dominant pattern. To further study this phenotype we performed cell culture experiments in which we controlled expression of various alleles in an otherwise genetically homogenous background (see Aim 2).

Specific Aim 2. Determine the antiestrogen response to OHT and RAL of cells in which wildtype and variant SULT1A1 and UGT1A6 allozymes have been expressed. These studies were designed to evaluate whether allele-specific differences in the response of cells to estrogens and antiestrogens could be measured. Sulfation of E2 and OHT results in inactive molecules (ie., they do not interact with the estrogen receptor). Therefore, we hypothesized that cells expressing the less active SULT1A1*2 allozyme would exhibit an enhanced proliferative response to E2 and OHT because those cells would have lower capacity to inactivate those molecules. Subaims were the following:

1) Generation of expression constructs for SULTs and UGTs
2) Generation of stably transfected human MCF-7 and HEK 293 cell lines

3) Cell proliferation assays
4) Quantitative RT-PCR of stably transfected MCF-7 cell lines
5) Correlation of UGT1A6 Genotype with phenotype in a bank of human liver tissues

We previously reported completion of these subaims. Our data revealed that cells expressing the low activity SULT1A1*2 allele proliferated significantly faster in response to treatment with the estrogen 17β-estradiol (E2) and significantly slower in response to the antiestrogen OHT. This was presumably because the capacity of cells expressing the low activity allozyme to inactivate these compounds was compromised. Further mechanistic analyses revealed that the *2 protein has a cellular half-life three to six -fold shorter than the wildtype protein, depending on the cell type studied (see previous progress reports). We have since evaluated the level of OHT sulfate formed in cells expressing SULT1A1*1 or SULT1A1*2. Our data suggest that OHT sulfate is completely transported to the extracellular media (a novel finding) and that less OHT sulfate is detected from cell culture plates expressing SULT1A1*2 than SULT1A1*1. Collectively our data suggest that a common genetic polymorphism in the human SULT1A1 gene (SULT1A1*2 allele) may be associated with greater estrogenic response of breast epithelial cells but may also be associated with greater antiestrogenic response of those cells to OHT.

We have generated HEK 293 cells stably expressing each of the four UGT1A6 allozymes. We have detected no correlation between response of those cells to OHT and UGT1A6 genotype. This result is likely due to the fact that OHT is a poor substrate for UGT1A6. However, we were able to confirm that cells expressing UGT1A6*1/*2 heterozygously exhibit significantly lower activity than cells expressing either allele alone (Fig 1). This confirmed that observation in human liver tissue.
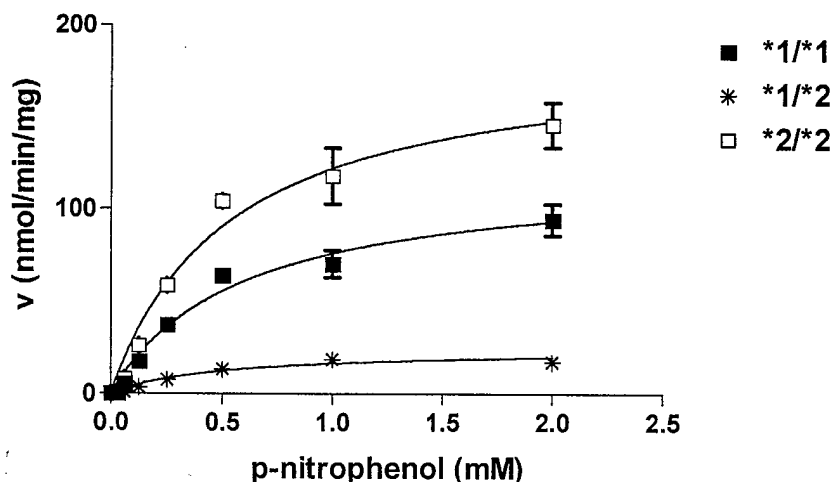

Fig 1. Michaelis – Menten kinetics of p-nitrophenol glucuronidation by homogenates of cells transiently expressing UGT1A6 *1/*1, *1/*2, and *2/*2 cells. Data represented as mean ± SD of triplicate measurements. Michaelis – Menten curves are representative curves drawn through mean data; parameter estimates were obtained by fitting the model to data replicates.

Specific Aim 3. Determine the association of SULT1A1, UGT1A6, CYP3A4 and CYP2D6 genotypes with the clinical response of women who are being prescribed TAM. The purpose of this aim was to determine the pharmacogenetic factors that impact clinical response to TAM. In previous progress

reports we presented that delays with approval of this clinical protocol by the Army led to a delay in the accrual of patients and necessitated a change in the consent form due to changes in the clinical prescribing of Tamoxifen. The amended our protocol seeks to study women taking tamoxifen for the adjuvant treatment of breast cancer. The amended protocol was approved only in June and we currently have ten subjects enrolled. This clinical study will continue without further financial support from the DOD.

KEY RESEARCH ACCOMPLISHMENTS

- Established HEK 293 cell lines stably expressing UGT1A6*1, *2, *3 and *4

- Compared kinetic properties of recombinant UGT1A6 allozymes

- Determined that low activity of the SULT1A1*2 allele is attributed to a combination of lower enzymatic "turnover" of substrate (low Vmax) as well as significantly shorter half life of the SULT1A1*2 protein compared to SULT1A1*1.

- Established expression profile of SULTs in human breast tumors and transformed breast carcinoma cell lines

- Developed TLC assay for evaluating OHT glucuronidation kinetics

- Developed high throughput genotyping assays for CYP3A4 and CYP2D6

REPORTABLE OUTCOMES (Bibliography of Publications)
Abstracts and Meetings

"UGT Pharmacogenetics, and Specificity of Raloxifene Glucuronidation", oral presentation, Jeffrey Zalatoris, DOD Era of Hope Meeting, Orlando, FL, September 2002.

"Common Alleles of Human Sulfotransferase SULT1A1 are Associated with Altered Biochemical and Cellular Response to Estrogens and Antiestrogens", poster presentation, DOD Era of Hope Meeting, Orlando, FL, September 2002.

"UDP-Glucuronosyltransferase-Specific Glucuronidation Inactivates 4-hydroxytamixfen and Raloxifene", Oral Presentation, 11th North American ISSX Meeting, Orlando, FL October 2002. Drig Metab Rev **34**:7, 2002.

"Pharmacogenetics of Human Sulfotransferases", Symposia Speaker, Annual FASEB meeting, San Diego, CA, April 2003.

"Functional Consequences of Individual Variation in Human SULT1A1 and UGT1A6", Symposia Speaker, *th Eurpoean ISSX Meeting, Dijon, France, April 2003.

Manuscripts

Swati Nagar*, Jeffrey J. Zalatoris* and Rebecca Blanchard. Human UGT1A6 Pharmacogenetics: Identification of a Novel SNP, Characterization of Allele Frequencies and Functional Analysis of Recombinant Allozymes in Human Liver Tissue and in Cultured Cells, *Pharmacogenetics*, in press. (* indicates that these authors contributed equally to this work).

Susan E. Walther, Amanda Thistle and Rebecca Blanchard, Association of SULT1A1 pharmacogenetics with cellular response to estrogens and antiestrogens, *in preparation*

CONCLUSIONS
In summary, we largely achieved the aims set forth in this grant. Specifically we have determined the SULT1A1 and, to a lesser extent, UGT1A6 contribute to the conjugative metabolism of OHT, the active metabolite of TAM and that UGT1A9 is predominantly responsible for RAL glucuronidation. Furthermore, we have identified and determined the allele frequencies for four common UGT1A6 alleles in ethnically defined human populations and have determined genotype/phenotype relationships for those alleles in a bank of human liver tissues. We have also studied each of the four recombinant UGT1A6 allozymes and collectively our data suggest that the *2 allozyme is associated with two-fold higher activity while it is associated with lower activity when expressed heterozygously with UGT1A6*1. We have developed a cell model system to study SULT1A1 and UGT1A6 allele-specific cellular phenotypes and have implemented that system to identify allele-specific proliferative response of cells to estrogens and antiestrogens. Specifically, we have determined that cells expressing the SULT1A1*2 variant respond to those compounds significantly more than cells expressing the SULT1A1*1 allele. The mechanism for this phenotype appears to be attributable to both altered enzyme kinetics as well as altered intracellular protein stability. Approximately 30% of Caucasians and African Americans are homozygous for the SULT1A1*2 allele. These results are significant in that should these allele-specific responses also occur in tumors -- SULT1A1 genotype might be associated with clinical response to tamoxifen.

REFERENCES

None

LIST OF PERSONNEL

Rebecca Blanchard Raftogianis – Principal Investigator
Susan Walther – Science Technician II
Andre Rogatko – Biostatistician
Marjon Vanslegtenhorst – Postdoc Associate
Jarrad Weikel – Summer Assistant II
Leslie Carlini – Research Associate

APPENDICES

Swati Nagar*, Jeffrey J. Zalatoris* and Rebecca Blanchard. Human UGT1A6 Pharmacogenetics: Identification of a Novel SNP, Characterization of Allele Frequencies and Functional Analysis of Recombinant Allozymes in Human Liver Tissue and in Cultured Cells, *Pharmacogenetics*, in press. (* indicates that these authors contributed equally to this work).

Human UGT1A6 Pharmacogenetics: Identification of a Novel SNP, Characterization of Allele Frequencies and Functional Analysis of Recombinant Allozymes in Human Liver Tissue and in Cultured Cells.

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Running Title: Human UGT1A6 pharmacogenetics

Keywords: UGT1A6, pharmacogenetics, allele frequencies

ABSTRACT

Background- UDP-glucuronosyltransferase (UGT) enzymes catalyze the glucuronidation and typically inactivation of endogenous and exogenous molecules including steroid hormones, bilirubin and many drugs. The UGT1A6 protein is expressed predominantly in liver and metabolizes small phenolic drugs including acetaminophen, salicylates and many β-blockers. Interindividual variation in the capacity of humans to glucuronidate drugs has been observed.

Results- We have identified a novel common single nucleotide polymorphism (SNP) in the human UGT1A6 gene that results in a Ser7Ala change in encoded amino acid. We have further functionally characterized that polymorphism in the context of two previously reported polymorphisms, Thr181Ala and Arg184Ser. These non-synonymous cSNPs define four common haplotypes. Alleles appear with similar frequencies in Caucasian and African-American populations with allele distributions adhering to Hardy-Weinberg equilibrium. UGT1A6 genotype, rate of substrate glucuronidation and level of immunoreactive UGT1A6 protein was determined. A 25-fold variation in the rate of substrate glucuronidation and an 85-fold variation in level of immunoreactive protein were measured. Liver tissue samples that were homozygous for UGT1A6*2 exhibited a high rate of glucuronidation relative to tissues with all other genotypes. Biochemical kinetic studies of recombinant UGT1A6 expressed in HEK293 cells indicated that the UGT1A6*2 allozyme, expressed homozygously, had almost 2-fold greater activity toward p-nitrophenol than UGT1A6*1, and when expressed heterozygously (UGT1A6*1/*2) was associated with low enzyme activity.

Conclusions- These data suggest that common genetic variation in human UGT1A6 confers functionally significant differences in biochemical phenotype as assessed in human tissue and in

cultured cells expressing recombinant allozymes. This genetic variation might impact clinical efficacy or toxicity of drugs metabolized by UGT1A6.

INTRODUCTION

Pharmacogenetic variation in genes that encode drug metabolizing enzymes contributes to interindividual differences in drug efficacy and toxicity [1,2]. Here we report the identification and functional characterization of common genetic polymorphisms in the human UGT1A6 gene. Glucuronidation is an important pathway in the detoxification and elimination of many drugs and is catalyzed by a superfamily of membrane-bound UDP-glucuronosyltransferases (UGTs) [3]. At least 14 functional UGT enzymes have been identified in humans. These comprise 2 UGT gene families (UGT1 and UGT2), assigned on the basis of amino acid sequence homology [4]. Genetic variation has been reported for several UGT genes including UGTs 1A1, 1A6, 1A7, 2B4, 2B7 and 2B15 [5-11]. The UGT1A family is encoded by a single nested gene locus spanning 200 kilobases on chromosome 2q37 [12]. Alternative transcriptional initiation at eight of thirteen possible first exons, followed by conserved splicing to common 2nd-5th exons, results in expression of eight specific UGT1A family members (Fig. 1A) [13,14]. Common genetic variation reported for UGT1A family members (1A1, 1A6 and 1A7) has been entirely within the unique first exon, while rare mutations in the 2nd-5th exons have been associated with metabolic diseases such as Crigler-Najjar syndrome and Gilbert's disease [5-7].

The human UGT1A6 protein is 531 amino acids in length with the carboxyl-terminal 245 amino acids being encoded by the common exons 2 through 5 and the remainder by the unique first exon [15]. UGT1A6 is expressed in a variety of tissues including liver, bile duct, colon, stomach and brain and catalyzes the glucuronidation of small, planar phenols and primary amines [3,16,17] including acetaminophen, β-blockers and salicylates [6,18]. No UGT crystal structures have been published but several studies have suggested that the amino acids necessary

for aglycone substrate binding are encoded in the variable first exon while those necessary for binding the cosubstrate, uridine diphosphate glucuronic acid (UDPGA), are encoded predominantly in the common exons 2 through 5 [15]. UGT1A6, like other UGTs, appears to function as a dimeric or possibly tetrameric protein, and the dimerization domain has been mapped to the amino terminus of the protein [19-22]. UGT1A6 is a type I endoplasmic reticulum (ER) bound protein with the majority of the protein, including the active site, residing within the lumen. There is a single transmembrane domain, and a short C-terminal domain of the protein projects into the cytosol (Fig. 1B). There are at least three domains that traffic UGT1A6 to the ER with apparently cotranslational and posttranslational mechanisms of insertion [23-25]. Those domains include 1) an N-terminal signal sequence (amino acids 1-26); 2) a putative internal signal that has been roughly mapped to amino acids 141-240; and 3) a dilysine motif at the carboxyl-terminus.

The UGT1A6 polymorphisms described in this report reside within the N-terminal signal sequence (Ser7Ala) and the putative internal ER-localization signal (Thr181Ala and Arg184Ser) (Fig. 1B). The Thr181Ala and Arg184Ser polymorphisms have been previously described, and those authors reported data describing differences among kinetic properties of recombinant enzymes harboring those two amino acid changes [6]. The present manuscript adds significantly to that body of knowledge by identifying the Ser7Ala polymorphism, defining UGT1A6 alleles and allele frequencies in Caucasian Americans and African Americans, and describing biochemical characterization of native allozymes in human liver tissue and recombinant allozymes in cultured human cells. Interestingly, our data suggest that the UGT1A6*2 allele is associated with increased levels of enzyme activity when expressed homozygously but with low

activity when expressed heterozygously (UGT1A6*1/*2) in both human liver tissue and cultured cells. It has increasingly become evident that the study of gene alleles or haplotypes, rather than specific nucleotide or amino acid changes, allows for optimal evaluation of functional consequences of genetic variation [26,27]. This work represents a foundation for future studies addressing the clinical significance of UGT1A6 haplotypes.

MATERIALS AND METHODS

Chemicals and reagents

UDPGA, alamethicin, α-naphthol, α-naphthyl-glucuronide, p-nitrophenol, p-nitrophenyl-glucuronide and JumpStart Taq Polymerase were purchased from Sigma (St. Louis, MO). Nu-Sieve agarose and Gel Star Nucleic Acid Stain were purchased from Biowhittaker Molecular Applications (Rockland, ME). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). NuPAGE gels, DH5α bacterial cells, pCR2.1, pcDNA3.1, OptiMEM, and zeocin were purchased from Invitrogen (Carlsbad, CA). UGT1A6 polyclonal antibody and microsomes expressing recombinant human UGT1A6 protein were obtained from Gentest (Woburn, MA). The Access RT-PCR kit was obtained from Promega corporation (Madison,WI). The pBlueBac4.5 plasmid containing the UGT1A6 cDNA was kindly provided by R. Tukey (UCSD).

Human subjects

All human samples were collected with approval of the Fox Chase Cancer Center Institutional Review Board. DNA was isolated from peripheral blood collected from healthy participants through the Biosample Repository at the Fox Chase Cancer Center (115 Caucasians and 74 African Americans). Human liver samples were obtained from the Fox Chase Cancer Center Tumor Bank. A total of 69 human liver samples were collected from 58 individuals who had undergone liver resections at Fox Chase Cancer Center because of metastatic colon cancer (n = 45) or primary hepatocellular carcinoma (n = 13). Matched tumor and adjacent normal liver tissue were collected from 11 of the 58 donors for a total of 69 samples. The 58 tumor samples were used to perform genotype/phenotype correlation while the matched tumor/normal pairs

were used to evaluate tumor-specific differences in UGT1A6 phenotype. Fifty-six tissue donors were Caucasian, one was an African-American individual and one was Asian-American (Cambodian).

DNA was isolated by the method of John et al [28]. Briefly, anticoagulated whole blood (5-8 ml) or liver tissue was mixed with an equal volume of DNA isolation buffer (10 mM Tris, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$) and 120 µl of Nonidet P-40. Cell nuclei were isolated by centrifugation at 800 x g for 10 minutes and resuspended in 800 µl of nuclear lysis solution (10 mM Tris pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.5 M NaCl, 0.5% SDS, and 2 mM EDTA). DNA was purified by a standard phenol:chloroform extraction procedure followed by ethanol precipitation. DNA was resuspended in sterile water or 10 mM Tris, pH 8.0 and stored at 4 °C.

Identification of UGT1A6 genetic polymorphisms

UGT1A6 SNPs were identified using PCR amplification and sequencing of the human UGT1A6-specific first exon as well as the common UGT1A 2nd-5th exons. PCR amplification was performed with 50-100 ng of genomic DNA, 1x JumpStart *Taq* buffer, 50 µM dNTPs, 500 nM PCR primers (Table 1) and 1 unit of JumpStart *Taq* polymerase, in a 50 µl reaction volume. JumpStart Taq polymerase was activated with a 60 second 94 °C denaturation step followed by 34 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds and 72 °C for 90 seconds, and an additional 4 minutes at 72 °C. PCR primers were verified to be specific for UGT1A6 primers by amplicon size determination as well as comparison of amplified DNA sequence to other UGT1A family members. Amplified DNA was purified using the Qiagen PCR Purification Kit (Valencia, CA). Polymorphisms were identified following DNA sequencing of amplified products, and

comparison of DNA sequence between samples. DNA sequencing was performed at the Fox Chase Cancer Center DNA Sequencing Core Facility using dye terminator chemistry and analyzed on a Perkin Elmer ABI Biosystems Model 377 (Foster City, CA). Heterozygous SNPs were confirmed upon sequencing of the antisense strand. Compound heterozygous genotypes were confirmed by sub-cloning PCR products and sequencing at least three clones.

PCR-RFLP assay for genotyping UGT1A6

A modification of the PCR-RFLP assay described by Ciotti *et al.* was used for the detection of the 541A>G and 552A>C cSNPs in population studies [6]. Genomic DNA (50-100 ng) was mixed with JumpStart *Taq* buffer in a reaction mixture containing 50 μM dNTPs, 500 nM primers and 1 unit of JumpStart *Taq* polymerase. To characterize the novel 19T>G SNP, primers F(-53) and R184 (Table 1) were used to amplify Product 1. Primers F414 and R628 spanned the 541A>G and 552A>C SNPs and were used in the amplification of Product 2 (Figure 2). Those PCR amplifications were performed with the following steps: 45 seconds at 94 °C preceded 34 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 50 seconds, which was followed by 3 minutes at 72 °C. Amplified products were digested for 3 hours at 37 °C using the following enzymes: product 1 amplicons (237 bp) with *Hha*I to detect 19T>G and product 2 amplicons (215 bp) with *Nsi*I and *Bbv*I, separately, to identify the 541A>G and 552A>C polymorphisms, respectively (Fig. 2). Digested products were separated on 2% Nu-Sieve agarose gels containing Gel Star Nucleic Acid Stain. Genotypes that were ambiguous on the basis of electrophoresis were confirmed by DNA sequencing of the purified PCR amplicons. Amplified DNA that was homozygous for UGT1A6*1 and *2 was sub-cloned into the pCR2.1 vector for use as positive controls. Integrity of all subcloned PCR products was checked by DNA

sequencing. Each set of assays also included a negative control sample in which no DNA was added to the reaction mixture. Twenty samples were genotyped by both DNA sequencing and PCR-RFLP methods to verify the accuracy of the PCR-RFLP assay.

Expression of recombinant UGT1A6 allozymes

The UGT1A6 cDNA was sub-cloned from the pBlueBac4.5 construct (kindly provided by R.Tukey, UCSD) into a pcDNA3.1 vector using *EcoRV:Xho*I sites. UGT1A6 alleles *2, *3 and *4 were generated by QuikChange XL site directed mutagenesis following the basic amplification procedures and *Dpn*I treatment as instructed (Stratagene, La Jolla, CA). The UGT1A6*3 (19T>G) allele was initially generated from the original pcDNA3.1/UGT1A6*1 construct using primers F5 and R29 (Table 1). UGT1A6*4 (552A<C) was generated using the pcDNA3.1/UGT1A6*3 construct and primers F541 and R562. Finally the pcDNA3.1/UGT1A6*2 construct was generated from the pcDNA3.1/UGT1A6*4 construct with primers F531 and R557. The UGT1A6 cDNA and proximal vector sequences were fully sequenced for all constructs to ensure accurate allele sequences.

Human kidney carcinoma cells (HEK293) were engineered to stably express UGT1A6*1, *2, *3 or *4 allozymes. Cells were grown in DMEM containing 10% FBS, 1% l-glutamate, and penicillin/streptomycin (3.5 units/3.5 µg per ml). pcDNA3.1/UGT1A6 plasmids (2 µg) were transfected with 100 µl serum-free OptiMEM and 6 µl FuGENE 6 transfection reagents (Roche, Indianapolis, IN) and laid over HEK293 cells grown to 60% confluence. Cells were split 1:10, 1:20, and 1:50 after two days and grown in the presence of 0.6 mg/ml zeocin. Colonies of stably transfected cells were isolated and expanded. The level of UGT1A6 protein expression and

activity were measured in each clone, and clones with similar protein expression levels were selected for continuous protein expression and harvesting. A negative control cell line was also generated by stably transfecting an empty pcDNA3.1 plasmid. For each of the four allozymes of UGT1A6, 6-8 clones were expanded.

In separate experiments, HEK293 cells were transiently transfected with pcDNA3.1/UGT1A6 plasmids to generate cells expressing UGT1A6 *1/*1, *1/*2, and *2/*2. Cells were also transfected with empty pcDNA3.1 vector for an experimental negative control. Plasmid (2 µg) was transfected with 100 µl serum-free OptiMEM and 6 µl FuGENE 6 transfection reagent and laid over HEK293 cells grown to 60% confluence. For the generation of *1/*2 heterozygous cells, 1 µg of each plasmid was added. Four 60 mm plates were transfected for each genotype. At the end of 48 hours, cells from the 4 plates per group were pooled and the cell pellet was divided for RNA and protein isolation.

The transfected cells were analyzed for the expression of UGT1A6*1 and/or *2 using a one-step RT-PCR reaction followed by RFLP analysis. Total RNA was extracted with the standard Trizol RNA isolation protocol including treatment with DNase (Promega Corp., Madison WI). The RT-PCR reaction was performed using the Access RT-PCR system (Promega Corporation, Madison WI) and the F414 and R628 primers. GAPDH was amplified as a control. The RT-PCR was performed as follows: 45 minutes at 48 °C followed by 2 minutes at 94 °C. This was followed by 38 cycles of 30 seconds at 94 °C, 40 seconds at 56 °C and 1 minute at 68 °C. There was a final extension step at 68 °C for 4 minutes. The amplified products were

digested for 3 hours at 37 °C with NsiI to generate allele-specific digestion products. The digested products were separated on a 2% agarose gel containing Gel Star nucleic acid stain.

Preparation of human liver microsomes and cultured cell homogenates

Microsomes were isolated using the method of Franklin and Estabrook [29]. Human liver tissue samples (150-250 mg) were homogenized in 600 µl of microsome isolation buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.25 M sucrose) using a Polytron PT1200 (Brinkmann Instruments, Inc., Westbury, NY). Low speed centrifugation (10 minutes at 9000 x g) was used to clear cellular debris, and DNA was extracted from this pellet as described. Microsomes were isolated by ultracentrifugation of the 9000 x g supernatants using a TLA100.2 rotor in a TL-100 Beckman Ultracentrifuge (Fullerton, CA) for 55 minutes at 100,000 x g. Microsomes were washed once with 0.15 M KCl, centrifuged for 35 minutes at 100,000 x g, and resuspended in microsome isolation buffer using the Polytron PT1200. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA) using BSA as a standard. Aliquots of cytosolic and microsomal samples were stored at –80 °C. Cultured cells were homogenized with 10 mM Tris buffer (pH 7.4) containing 0.25 M sucrose in a Dounce homogenizer, and homogenates were stored at –80 °C.

Spectrophotometric assay

One measure of UGT1A6-associated phenotype was rate of glucuronidation of α-naphthol and, separately, p-nitrophenol in human liver microsomal preparations and with recombinant UGT1A6 proteins. Unfortunately, there is no one substrate that is unique for UGT1A6 among all UGT isoforms. However, considering the high level of expression of

UGT1A6 in the liver compared to other isoforms that would be expected to turn over these substrates at the indicated conditions, UGT1A6 is likely the predominant isoform contributing to the measured activities, particularly for α-naphthol [3].

High throughput assays were developed and were based on spectrophotometric loss of absorbance of the aglycone substrate upon glucuronidation [30]. Maximal absorbance for p-nitrophenol and α-naphthol was 402 nm and 332 nm, respectively. Substrate absorbance was linear with p-nitrophenol and α-naphthol concentration and was enhanced at alkaline pH. Therefore to optimally detect spectral changes of the quenched reaction products, the pH was adjusted to alkaline conditions (pH 12), and the absorbance was measured at 402 nm (p-nitrophenol) or 332 nm (α-naphthol). The assay was accurate and precise for the entire concentration range of either substrate. The limit of quantitation for p-nitrophenol was 6 μM while that for α-naphthol was 30 μM. The limit of detection for each substrate was 0.25 μM and 6 μM respectively. p-nitrophenol and α-naphthol glucuronides were stable at pH 12 for over 3 hours. Protein concentration and length of incubation were optimized to determine conditions over which a linear rate of substrate glucuronidation was observed. Reaction rates were linear at concentrations up to 0.5 mg/ml of microsomal protein up to 120 minutes for p-nitrophenol and to 40 minutes for α-naphthol. Glucuronidation rates in human liver microsomes were assayed at 0.3 mg/ml total protein at 37 °C for 40 minutes and 120 minutes for α-naphthol and p-nitrophenol, respectively. Assays were also optimized with regard to concentration of detergent. Both alamethicin and Tritox-X-100 were evaluated for maximal activation of liver microsomes. Optimal activation was achieved using 0.02% Triton X-100.

Liver microsomes were thawed on ice and added (at 0.3 mg/ml final concentration) to fresh assay buffer (25 mM Tris-HCl pH 7.5, 25 mM KCl, 10 mM $MgCl_2$, 0.02% Triton X-100) containing excess UDPGA (4 mM). A baseline sample (20 µl blank) from each reaction mixture was transferred to the quenching solution (5 µl of 10% perchloric acid and 150 µl water). Assays were initiated, in quintuplet, with 500 μM α-naphthol or p-nitrophenol as substrate and 2 reactions were immediately quenched and used as time zero control samples. The remaining reactions performed in triplicate were incubated at 37 °C in a 96-well assay plate. Reactions were quenched after 40 minutes (α-naphthol) or two hours (p-nitrophenol), and proteins were separated from reactants and reaction products upon centrifugation. Supernatants were transferred to a clean Costar 96-well UV plate and brought to alkaline pH with 20 µl of 0.75 M NaOH. Final sample volume was raised to 200 µl with water. Spectrophotometric absorption was measured at 332 nm to detect α-naphthol and 402 nm for p-nitrophenol on a SPECTRAmax PLUS Microplate Spectrophotometer (Molecular Dynamics, Sunnyvale, CA). Conversion of absorbance to rate of glucuronidation was based on linear regression analysis of the absorbance for multiple concentrations of native and glucuronidated standards prepared in the same reaction condition as the assays. Glucuronidation of the commercially available recombinant UGT1A6 was monitored with each set of experiments to evaluate assay variability and we observed negligible inter-plate variability.

Reactions were performed with the cell homogenates in triplicate using 4 mM UDPGA, p-nitrophenol concentrations from 0.05 – 2 mM, and 0.1 mg/ml microsomes containing heterologously expressed UGTs. Again, both alamethicin and Tritox-X-100 were evaluated for maximal activation of cell homogenates. Optimal activation was achieved using alamethicin at a

concentration of 25 µg /mg total protein. Reaction rates were normalized based on Western blot analysis of immunoreactive UGT1A6 in HEK293 cell lysates. No significant background glucuronidation of p-nitrophenol or immunoreactive UGT1A6 was detected in mock-transfected HEK293 cells.

Western blot analyses

The level of immunoreactive UGT1A6 protein was determined in 52 of the tumor tissue samples and 10 normal samples and in the stably and transiently transfected cells. Fifty µg of human liver microsomal protein or cell homogenate were electrophoresed on NuPAGE 10% SDS-polyacrylamide gels and transferred to Immobilon P membranes (Millipore). Membranes were blocked in 5% milk in Tris-buffered saline with 0.05% Tween-20 (TTBS) for 3 hours and incubated with anti-UGT1A6 primary antibody (1:5000, Gentest) for 16 hours. Membranes were washed in TTBS, followed by a 2-hour incubation in HRP-conjugated goat anti-rabbit antibody (1:10,000, BioRad, Hercules, CA). Membranes were washed in TTBS and Tris-buffered saline (TBS) and proteins were detected using the Amersham Pharmacia (Piscataway, NJ) ECL chemiluminescent substrate. The film image was digitized using Kodak Image Station 440 with image processing and data analysis was performed on Kodak 1D Image Analysis 3.0 software (New Haven, CT). On each gel, three concentrations of a recombinant UGT1A6 standard (Gentest, 1 relative unit = 1 µg) were included and evaluated by linear regression, which was used to quantify UGT1A6 protein level in liver samples. Level of immunoreactive UGT1A6 protein was reported per µg of total microsomal or cell homogenate protein. This method of normalization allowed for direct comparison between level of immunoreactive protein and level of UGT activity.

Statistical analyses

The Hardy-Weinberg Diagnostics program HWDIAG v. 1.0β (http://www.fccc.edu/users/rogatko/hwdiag/user_guide.html) was used to determine equilibrium of the UGT1A6 genotypes within healthy Caucasians and African-Americans with the limit of significance set at 0.05 [31]. The program performs multiple simulations for a population data set using classical type II error to determine if the population distribution fits an equilibrium state. Nucleotide linkage was analyzed by Fisher's exact test for independence for each nucleotide pair and stratified by race. For the comparison of genotype and phenotype of liver tissues, ANOVA followed by Tukey's comparison test was performed using the GraphPad Prism 3.0b software (GraphPad Software, Inc., San Diego, CA) with $p < 0.05$ as the limit of significance. Correlation was determined using the two-tailed Pearson's test (Prism).

RESULTS

Four UGT1A6 alleles were identified

Four single nucleotide polymorphisms were identified among 40 healthy Caucasians: 19T>G, 315A>G, 541A>G and 552A>C (Table 2). The 315A>G polymorphism is a synonymous SNP, coding for a leucine at amino acid 105. The other three polymorphisms were non-synonymous cSNPs encoding amino acid changes of Ser7Ala, Thr181Ala and Arg184Ser. The Ser7Ala polymorphism has not previously been reported. No genetic variation in the common exons was observed in this population. Therefore, common haplotypes were defined entirely by genetic variation in the first exon. Permutations of the SNPs in exon 1 defined four distinct alleles (Table 2). Pairwise analyses of the 3 non-synonymous cSNPs using Fisher's exact test supported the conclusion that these SNPs significantly associate as distinct haplotypes (for each pairwise test, $p < 0.0001$).

Following the identification of UGT1A6 alleles, a rapid method for genotyping was employed (Fig. 2). A total of 115 healthy Caucasian and 74 healthy African-American blood donors were genotyped to determine UGT1A6 allele frequencies. No additional ethnic-specific UGT1A6 polymorphisms were detected after completely sequencing all UGT1A6 exons from 10 African-American samples. UGT1A6*1, *2, *3 and *4 alleles were defined on the basis of allele frequencies as shown in Table 2. Allele distribution followed Hardy-Weinberg equilibrium in both the Caucasian and African American populations ($\alpha^* = 0.08$ and 0.74, respectively, where $\alpha^* > 0.05$ represents a significant probability of adhering to Hardy-Weinberg equilibrium).

Hepatic UGT1A6 activity displayed significant interindividual variation

The rate of substrate glucuronidation was determined in liver tumor tissue from 58 individuals. Among these samples, the rate of α-naphthol glucuronidation correlated with the rate of p-nitrophenol glucuronidation ($r^2 = 0.402$, $p < 0.0001$). There was an overall 25-fold variation in the rate of substrate glucuronidation among liver tissues. The range of activities toward p-nitrophenol was 0.387 to 9.64 nmol/min/mg protein and that of α-naphthol was 1.18 to 27.28 nmol/min/mg protein (Fig. 3). We also measured the level of immunoreactive UGT1A6 protein in 52 liver tumor microsomes (Fig. 4A). There was 85-fold variation in the level of UGT1A6 protein among all tissues studied. The rates of p-nitrophenol and α-naphthol glucuronidation were each positively correlated with level of immunoreactive protein ($r^2 = 0.324$, $p < 0.001$, $r^2 = 0.242$, $p < 0.001$, respectively).

UGT1A6 genotype correlates significantly with UGT1A6 phenotype

The distribution of UGT1A6 genotypes in liver tissues followed Hardy-Weinberg Equilibrium similar to the distribution in the population of healthy Caucasian blood donors (α* = 0.08 for Caucasian blood donors and 0.11 for liver tumor tissues, which were predominantly from Caucasians). Though beyond the scope of this manuscript, it is of potential interest that tissues with the *2/*2 genotype were underrepresented by a factor of 2 among tumor tissues. The most significant observation was that samples homozygous for UGT1A6*2 exhibited the greatest rate of glucuronidation compared to tissues with other genotypes (Fig. 3). The mean glucuronidation rates for tissues with the *2/*2 genotype were 27.3 ± 1.48 nmol/min/mg protein for α-naphthol and 9.64 ± 1.47 nmol/min/mg protein for p-nitrophenol. In contrast, the mean glucuronidation rates for other genotype groups were 13.4 ± 2.79 ($p < 0.05$) and 4.13 ± 0.98

nmol/min/mg protein ($p < 0.05$) for *1/*1, 9.35 ± 1.23 ($p < 0.05$) and 1.90 ± 0.40 nmol/min/mg protein ($p < 0.01$) for *1/*2 and 1.18 ± 1.18 ($p < 0.05$) and 0.39 ± 0.31 nmol/min/mg protein ($p < 0.05$) for *1/*3 (α-naphthol and p-nitrophenol, respectively). Only one tissue with *1/*4 genotype was available, which exhibited activity toward α-naphthol (13.2 nmol/min/mg) but not p-nitrophenol.

The level of immunoreactive UGT1A6 protein was evaluated in each microsomal sample by Western blot analysis (Figs. 4A and B). UGT1A6 protein levels correlated with level of p-nitrophenol glucuronidation activity within the *1/*1 and *1/*2 genotype groups ($r^2 = 0.510$, $p < 0.01$ and $r^2 = 0.571$, $p < 0.001$, respectively). When glucuronidation rate data were normalized to level of immunoreactive protein, variation in rate between genotype groups was significantly diminished, suggesting that one possible mechanism by which genotype influences UGT1A6 function is through altered levels of steady state protein (Fig. 4C). A notable exception to this trend is the sample with *1/*4 genotype. This sample exhibited no activity toward p-nitrophenol, moderate activity toward α-naphthol, but the highest level of immunoreactive protein among all tissues studied (Figs. 3 and 4B). With only one sample, we were unable to evaluate whether this was genotype specific. Overall these results indicate that genotype was associated with rate of glucuronidation within liver tumor tissue (Fig. 3) and that genotype-specific variation was in part due to differences in levels of immunoreactive protein (Fig. 4B).

To corroborate the glucuronidation rate data from human liver tissues and to measure the kinetics of glucuronidation by all four UGT1A6 allozymes, recombinant allozymes were stably expressed in HEK293 cells. Western blots of subcellular fractions from each stably transfected

cell line indicated that immunoreactive UGT1A6 protein was exclusively localized to microsomes and not to the cytosolic fraction. UGT1A6*1 exhibited the lowest specific activity among all allozymes, having the highest apparent K_M value and the lowest apparent V_{max} (Table 3). Note that the approximately two-fold higher specific activity for recombinant UGT1A6*2 is in agreement with the level of activity in human liver tissue expressing UGT1A6*2 homozygously. Table 3 also suggests that the specific acitivities of the *3 and *4 allozymes are high, however this phenotype could not be corroborated by human liver tissue due to lack of samples expressing these allozymes homozygously. The negative control HEK293 cells did not express UGT1A6 protein nor exhibit glucuronidation activity toward p-nitrophenol.

Cells expressing UGT1A6*1/*2 (heterozogously) exhibit a non-codominant, low activity phenotype

A surprising observation in the human liver data was that tissue expressing UGT1A6*1/*2 heterozygously exhibited lower activity than tissue expressing either allele homozygously (Fig. 3). To study this phenotype in an independent experimental system, we transiently transfected HEK293 cells with plasmids encoding for UGT1A6*1, *2 or both *1 and *2 to mimic homozygous or heterozygous status. Figure 5A demonstrates expression of the expected UGT1A6 transcripts (*1, *2 or both *1 and *2) in transiently transfected cells. Cell homogenates were evaluated for enzyme activity towards p-nitrophenol. Figure 5B demonstrates that the kinetic data generated in this experimental system very nicely corroborated the data observed in human liver tissue (Fig. 3). Cells expressing *2 homozygously exhibited the highest activity, followed by those expressing *1, while cells expressing both *1 and *2 had the lowest

activity. Mock-transfected cells exhibited no enzymatic activity. The Michaelis – Menten parameter estimates for each genotype were as follows:

a) *1 / *1: V_{max} = 120 ± 9 nmol/min/mg (estimate ± SE); K_M = 0.58 ± 0.1 mM; V_{max}/K_M = 207 ml/min/mg

b) *1 / *2: V_{max} = 25 ± 2 nmol/min/mg; K_M = 0.49 ± 0.09 mM; V_{max}/K_M = 50 ml/min/mg

c) *2 / *2: V_{max} = 186 ± 16 nmol/min/mg; K_M = 0.53 ± 0.1 mM; V_{max}/K_M = 352 ml/min/mg

Hepatic UGT1A6 phenotype is not tumor specific

We had the opportunity to compare UGT1A6 phenotypes in eleven matched tumor/normal tissue samples to determine the extent to which phenotypes in diseased tissue deviated from that of normal tissue. To evaluate tumor-dependent UGT1A6 activity, rate of glucuronidation and level of immunoreactive protein were compared in the paired normal/tumor tissue sets. Tumors were either primary hepatocellular carcinoma (n = 3) or metastases of primary colorectal tumors (n= 8). For all matched tissue pairs, the genotypes were concordant: three were *1/*1, seven were *1/*2, and one was *2/*2, suggesting no tumor-associated loss of heterozygosity. Glucuronidation rates from liver tumors were 15.0 ± 3.05 and 3.71 ± 1.16 nmol/min/mg protein for α-naphthol and p-nitrophenol, and the rates from the adjacent normal tissues were 12.1 ± 2.57 and 5.29 ± 0.87 nmol/min/mg, (p = 0.215, p = 0.247) respectively. Furthermore no significant differences in rate of glucuronidation or level of immunoreactive UGT1A6 protein were noted between primary tumors (n = 12) or those that were metastatic (n = 40).

DISCUSSION

In this study, we identified four common UGT1A6 alleles in healthy Caucasian and African-American populations. UGT1A6 genotype correlated with the level of UGT1A6-associated activity and immunoreactive protein in a bank of archived human liver tissues. We observed no gender differences in level of protein expression or UGT activity. However, this study was not designed to explicitly evaluate gender differences in expression and activity. Increased levels of UGT1A6 protein have been reported in male livers compared with female livers [32]. Additionally, functional variation was confirmed in kinetic studies using recombinantly expressed UGT1A6 allozymes. Our data indicated that functional differences were associated with common UGT1A6 allozymes, and several molecular mechanisms may contribute to these differences.

The three common cSNPs that defined the alleles encoded Ser7Ala, Thr181Ala, and Arg184Ser amino acid changes. Sequence comparison demonstrated that Ala at amino acid 7 is conserved in UGT1A6 among species but not within other human UGT1A isoforms. This amino acid resides within the N-terminal ER signal sequence of the protein. The amino acid at residue 181 is not conserved between species or among human isoforms. Amino acid 184 is not conserved in UGT1A6 among species but is a conserved glutamine in all other human UGT1A isoforms. This analysis suggests that amino acid sequence at position 184 may be important for human UGT1A specific phenotypes. The Ser7Ala polymorphism may contribute to increased enzyme activity, since all the allozymes with this polymorphism (*2, *3, and *4) showed higher intrinsic activity than *1 (table 3). Interestingly, the additional Ala181Thr polymorphism in *4 compared to *2 further increased the intrinsic activity of the enzyme. Determination of amino

acids critical for UGT catalytic activity is in its infancy but no reports have indicated that any of the polymorphic amino acids in UGT1A6 are critical for substrate binding. However, amino acids 181 and 184 map to a domain that is highly variable between UGT isoforms and is thus predicted to contribute to substrate specificity [15].

A previous study of UGT1A6 polymorphisms reported the Thr181Ala and Arg184Ser variants [6]. Those authors determined that recombinant UGT1A6*1 (Ser7/Thr181/Arg184, which we identified as the most common allele) was more active than recombinant Ser7/Ala181/Ser184 (which is an allozyme that we did not observe in 516 alleles studied) using an *in vitro* biochemical assay. However, the Ser7Ala variant was neither reported nor evaluated by those authors, and genotype/phenotype correlation in human tissue was not addressed in that study. It is understood that the leader sequence containing Ser7 is not present in the mature UGT1A6 protein; however, it has been suggested to be important for proper insertion of the protein into the endoplasmic reticulum. Our data suggest that the Ala7/Ala181/Ser184 allozyme (UGT1A6*2) was actually more active (in the homozygous state) than the wild-type allozyme in microsomes isolated from human liver tissue and HEK293 cells expressing recombinant proteins. The discrepancy between the data from Ciotti *et al.* and the results reported here may be a partial reflection of different reaction conditions: pH, buffer composition, protein source (tissue homogenates versus microsomes), protein concentration, and substrate concentrations. It is also possible that the discrepancy reflects differences attributable to the Ser7Ala amino acid change, which resides in a putative ER signal sequence.

In the current work, p-nitrophenol and α-naphthol were used as substrates for UGT1A6. Phenols are in fact known to be substrates for UGT1A9 as well as UGT1A6 [33]. Although these are not specific substrates, they have been widely used as standard measures of UGT1A enzyme activity, of which UGT1A6 is a major contributor [34,35,3]. Recently, serotonin has been suggested as a specific probe for UGT1A6 [36]. Future studies will examine the glucuronidation of serotonin by various UGT1A6 allozymes.

We observed differences in rate of glucuronidation among tissues that were homozygous for the *1 allele, homozygous for the *2 allele or heterozygous for these alleles. However, the differences that we observed were not consistent with classical models of co-dominance. Based on co-dominance of alleles toward phenotypic expression, one would predict that tissues that are heterozygous would have UGT1A6 activity intermediate to the activities of tissues that were homozygous for either allele. However, we observed that samples with heterozygous expression of *1/*2 exhibited lower activity than tissues homozygous for *1 or *2 (Fig. 3). These heterozygous samples also displayed a lower level of immunoreactive microsomal protein (Fig. 4B). This trend was in fact also observed in transiently transfected HEK293 cells, where again cells expressing *1/*2 exhibited much lower activity than those expressing either *1 or *2 homozygously (Fig. 5B). It is interesting to consider the possibility that *1/*2 protein heterodimers may have lower activity because of perturbation of localization within the ER membrane, protein dimerization or protein stability. We examined cytosolic fractions of liver samples as well as the HEK293 cells expressing different allozymes and found no localization of UGT1A6 to the cytosol. Studies are currently under way to characterize heteroalleles of UGT1A6 other than *1/*2 in order to fully understand these results.

A dominant negative model for UGT heterodimers was recently supported by data showing that heterodimers of synthetic mutants in a related protein, UGT1A1, exhibited rates of glucuronidation lower than predicted [22]. Further, in two reports examining molecular mechanisms of the hyperbilirubinemic diseases Gilbert's Syndrome and Crigler-Najjar Syndrome II, a dominant negative model was proposed to describe the exacerbated reduction of UGT activity in individuals with heterozygous UGT1A1 mutations [37,38]. Considering that each of the polymorphisms defining the *2 allele alter encoded amino acids within putative ER localization domains, it is possible that localization within the ER and/or dimerization at the ER is affected. That hypothesis will be addressed in future experiments.

Our data suggest that the amino acid variants that define common UGT1A6 allozymes might contribute to altered intrinsic catalytic properties of the enzymes as well as the level of expression of the proteins (Fig. 4 and Table 3). The expression levels of UGT1A6 in this study were determined with Western blot analysis. The antibody used for these analyses does not react with other UGTs to the best of our knowledge. However, it is not known whether this antibody immunoreacts to the same extent with all polymorphic forms of UGT1A6. Any difference in its immunoreactivity towards various polymorphs would be a potential limitation of this study. Differences in level of protein may be a reflection of differences in allozyme or dimer-specific protein stability, or linkage with as yet unidentified polymorphisms in regulatory elements of the UGT1A6 gene. Those possibilities will be addressed in future studies.

Lastly, we evaluated the possibility that UGT1A6 genotype distribution, UGT1A6 activity or level of protein might differ between liver tumor tissue and adjacent "normal" tissue. The insignificant differences in glucuronidation rates by tumor and matched normal tissues suggest that UGT1A6 activity is neither up nor down regulated in transformed hepatic or metastasized colorectal tissues. We noted deviation from equilibrium for UGT1A6 genotype in subjects with cancer that was attributable to an under-representation of *2 homozygous genotypes and over-representation of *1/*2 heterozygous genotypes. These results may be a reflection of differences in the sample populations, although an association between colorectal cancer and UGT1A6 genotype cannot be excluded. In the matched tumor and adjacent tissue pairs, no significant differences were detected for the level of protein expression (n= 10 pairs) or rate of substrate glucuronidation (n= 11 pairs) when comparing different genotypes or when comparing between metastatic and primary tumors. UGT1A6 expression has been compared in normal vs. diseased tissue in other studies. Work by Strassburg et al. found no difference in the expression of UGT1A6 in matched normal and gastric adenocarcinoma tissue for 14 tissue sample pairs [39]. Another study reported a lack of difference in UGT1A6 expression in normal versus tumorous pharyngeal mucosa [40].

In conclusion, because UGT1A6 is highly expressed in liver and an enzyme important in the metabolic disposition of many drugs, we examined the association between UGT1A6 genotype and UGT1A6-associated phenotypes in human liver microsomes. A 25-fold variation in rate of glucuronidation was detected in a bank of human liver samples and that variation was in part attributed to genotype (Fig. 3). Our data suggest that altered catalytic properties are associated with common UGT1A6 sequence variation and may contribute to allozyme-specific

phenotypes. Further experimentation is required to address the clinical relevance of these polymorphisms. The work described here provides a foundation for future studies addressing the association between UGT1A6 genotypes and clinical response to drugs.

ACKNOWLEDGMENTS

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Table 1. **UGT1A6-Specific Oligonucleotide Primers**

Primer Name	Reactions	Sequence
F(-207)	Amplify and Sequence Exon 1	5' GCC ATA GTT GGT TCA TAT TAA CCA TGT GAT 3'
R1I93		5' CTC CAA GGG ACA GTC GGC TCA A 3'
F473	Sequence Exon 1	5' TCC TGG CTG AGT ATT TGG GCC 3'
R589		5' TGT AGC ACC TGG GAA TGT AGG AC 3'
I1F(-118)	Amplify and Sequence Exon 2	5' AAA CAC GCA TGC CTT TAA TCA TAG T 3'
I2R109		5' AAA AGC CAA ATC TAA GGT TCC AAT TA 3'
I2F(-84)	Amplify and Sequence Exons 3-4	5' ACC CTG TTT TCT AGT TAG TAT AGC 3'
I4R49		5' ATC ATG AAT GCC ATG ACC AAA G 3'
I3F169	Sequence Exon 4	5' TCT GCA AGG GCA TGT GAG TAA C 3'
I4F(-112)	Amplify and Sequence Exon 5	5' CCT TTC CCA AGT TTG GAA AAT CTG GTA GT 3'
DR267		5' CAG CCT GTC TGC ACG TCC TCT G 3'
F(-53)	PCR-RFLP for 19T>G	5' GAT TTG GAG AGT GAA AAC TCT TT 3'
R184		5' CAG GCA CCA CCA CTA CAA TCT C 3'
F414	PCR-RFLP for 541A>G, 552A>C	5' CTT TAA GGA GAG CAA GTT TGA TG 3'
R628		5' CCA CTC GTT GGG AAA AAG TC 3'
F5	Mutation of 19T>G	5' CCT GCC TCC TTC GC<u>G</u> CAT TTC AGA G 3'
R29		5' CTC TGA AAT G<u>C</u>G CGA AGG AGG CAG G 3'
F531	Mutation of 541A>G	5' CCT GGA GCA T<u>G</u>C ATT CAG CAG AAG CCC 3'
R557		5' GGG CTT CTG CTG AAT G<u>C</u>A TGC TCC AGG 3'
F541	Mutation of 552A>C	5' ACA TTC AGC AG<u>C</u> AGC CCA GAC C 3'
R562		5' GGT CTG GGC T<u>G</u>C TGC TGA ATG T 3'

Table 2   **Common nucleotide and amino acid variations of the UGT1A6 gene**

Allele	19	(7)	315	(105)	541	(181)	552	(184)	Caucasian		African-American	
	Nucleotide (Amino Acid)								Allele Frequencies (Number)			
*1	T	(Ser)	A	(Leu)	A	(Thr)	A	(Arg)	0.617	(142)	0.608	(90)
*2	G	(Ala)	G	(Leu)	G	(Ala)	C	(Ser)	0.274	(63)	0.243	(36)
*3	G	(Ala)	A	(Leu)	A	(Thr)	A	(Arg)	0.057	(13)	0.10	(15)
*4	G	(Ala)	G	(Leu)	A	(Thr)	C	(Ser)	0.052	(12)	0.047	(7)

Allele frequencies were determined by either DNA sequencing or application of the PCR-RFLP assay in a total of 115 healthy Caucasian and 74 healthy African-American subjects.

Table 3   **Biochemical parameters for recombinant UGT1A6 allozymes**

Allozyme	K_M (mM)	V_{max} (nmol/min/mg)	V_{max}/K_M (ml/min/mg)	Variation from *1 (Ref)
*1	1.1 +/- 0.23	179 +/- 18	161.3	Reference
*2	0.73 +/- 0.15	219 +/- 19	300.0	1.86x
*3	0.57 +/- 0.29	283 +/- 58	496.5	3.08x
*4	0.45 +/- 0.16	249 +/- 31	553.0	3.43x

Substrate concentration was 0.050-4.0 mM p-nitrophenol; cosubstrate UDPGA concentration was 4.0 mM.

Legends for Figures

Figure 1. (A) The 200 kilobase UGT1A gene locus (Accession #AF297093) is depicted with unique first exons (boxes) and four common exons (lines labeled 2-5). Pseudogenes ("p") are designated with diagonally-hatched boxes. Actively transcribed first exons (black boxes) are alternatively spliced to the 2nd-5th exons, encoding unique UGT1A proteins. (B) The UGT1A6 protein structure includes two ER-targeting signal sequences, two glycosylation sites, a transmembrane domain and a cytosolic carboxy-terminus. The three polymorphic amino acids (annotated with the more frequent amino acids) are located in ER-targeting domains.

Figure 2. UGT1A6 PCR-RFLP genotype assay. (A) Separate amplification of segments of the UGT1A6 first exon was performed with genomic DNA as template (Table 1). (B) Resulting amplicons (Product 1; 237 bp and Product 2; 215 bp) were digested by *Hha*I, *Nsi*I or *Bbv*I to diagnose the 19T>G, 541A>G and 552A>C polymorphisms, respectively. (C) Diagnostic restriction patterns.
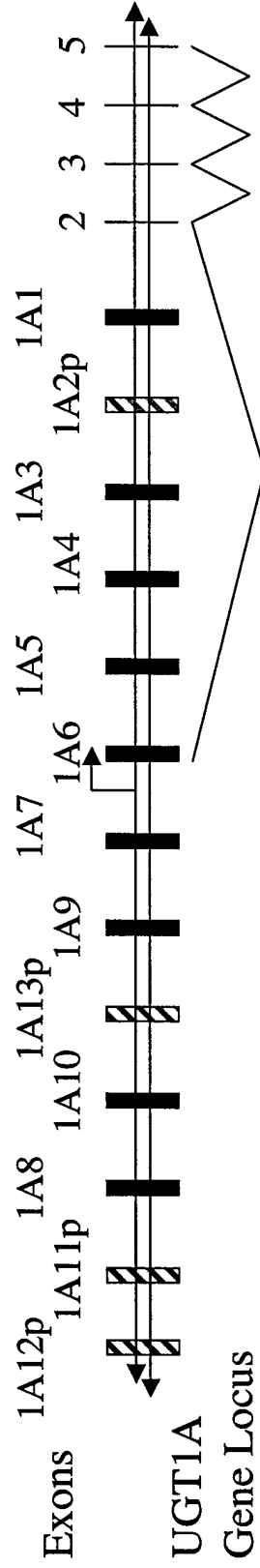
Figure 3. Genotype/phenotype correlation for UGT1A6 alleles in a bank of human liver tissues. Mean glucuronidation rates ($\pm$ SEM) of α-naphthol and p-nitrophenol are depicted stratified by genotype. The number of liver microsomes within each genotype group is listed above error bars. ANOVA followed by Tukey's comparison test was performed for all genotype groups.

Figure 4. (A) Level of UGT1A6 immunoreactive protein was analyzed by Western blot. 50 μg of microsomal protein were separated by gel electrophoreses. Immunoblots were probed with the α-WB-UGT1A6 primary antibody (Gentest). For each gel, three concentrations of

recombinant UGT1A6 standards (1 Unit = 1 μg) were also included to generate a standard curve for quantification of immunoreactive UGT1A6. Sample identification numbers are depicted above lanes containing liver microsomes ("N", normal and "T", tumor). Genotypes of samples are: *1/*1 for 380N, 380T and 204T; *1/*2 for 978T and 455T; *2/*2 for 053T. (B) Level of immunoreactive UGT1A6 protein stratified by UGT1A6 genotype. Mean densitometry units ($\pm$ SEM) are depicted for each genotype group. Due to low number of the *2/*2 tissues, differences of mean protein levels were not statistically significant ($p > 0.05$). (C) Glucuronidation rates for liver microsomes were normalized to level of immunoreactive UGT1A6 based on densitometry. Bars represent the mean normalized rate.
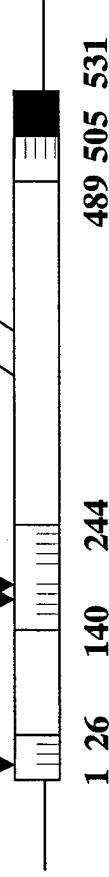
Figure 5. (A) RT-PCR – RFLP analysis of HEK293 cells that were mock-transfected (pcDNA3.1) or transiently expressing UGT1A6 *1/*1, *1/*2, or *2/*2. Total RNA from cell pellets was used to produce and amplify cDNA, which was digested with NsiI. The digested products were separated on a 2% agarose gel. (B) Michaelis – Menten kinetics of p-nitrophenol glucuronidation by homogenates of cells transiently expressing UGT1A6 *1/*1, *1/*2, and *2/*2 cells. Data represented as mean $\pm$ SD of triplicate measurements. Michaelis – Menten curves are representative curves drawn through mean data; parameter estimates were obtained by fitting the model to data replicates.

A)

Exons
1A12p 1A8 1A13p 1A7 1A5 1A3 1A1
1A11p 1A10 1A9 1A6 1A4 1A2p
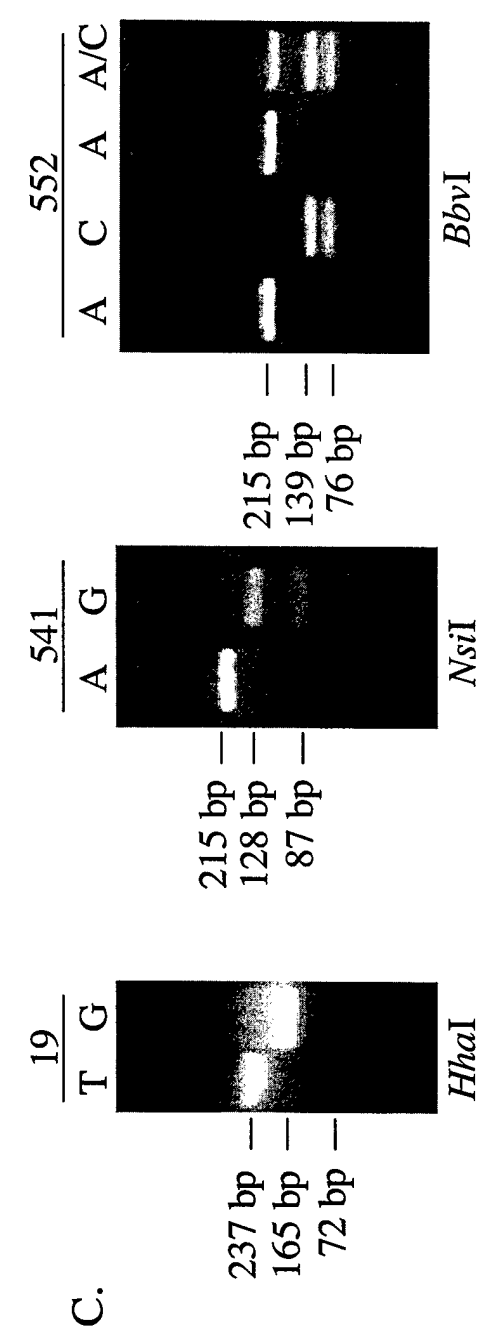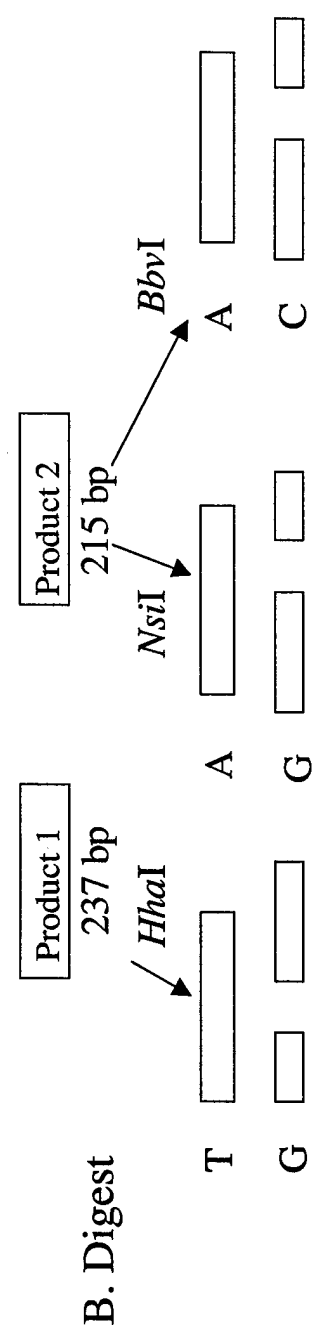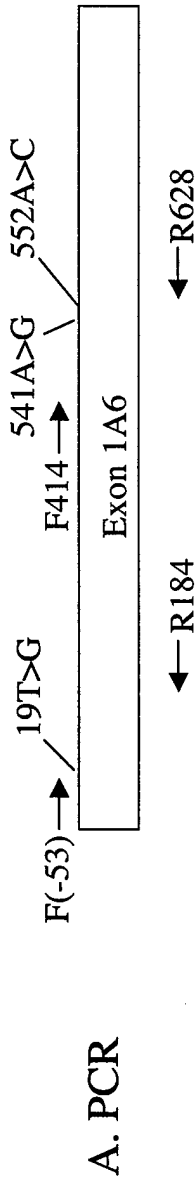
UGT1A
Gene Locus

Exons: 2 3 4 5

▼ 3 non-synonymous cSNPs
▢ ER signal domains
▤ Transmembrane domain
■ Cytosolic tail
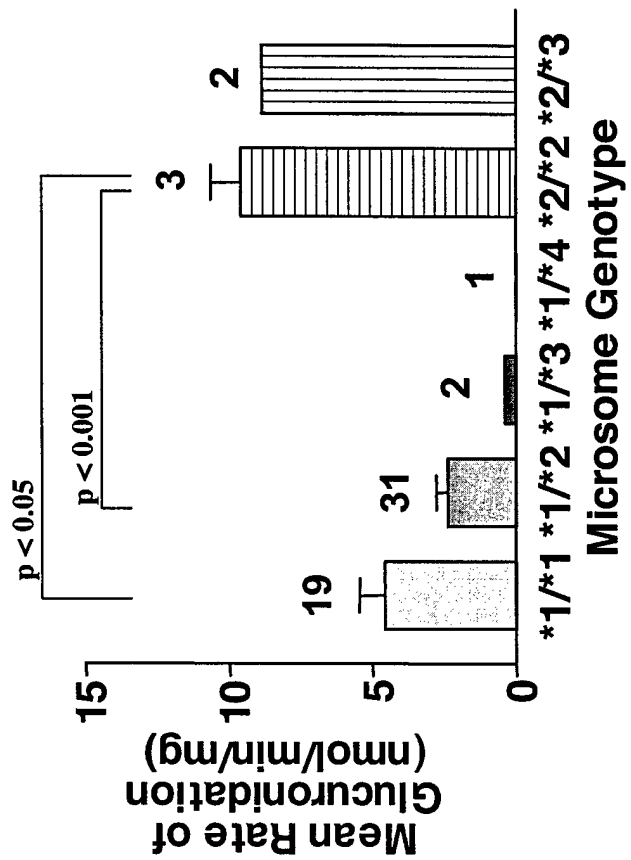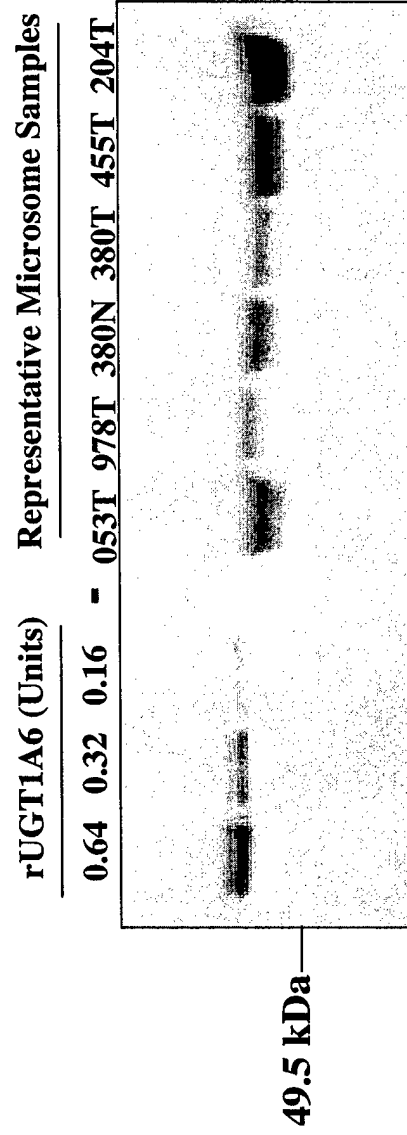γ N-linked glycosylation sites

B)

Ser7 Thr181 Arg184 Asn293 Asn345

1 26 140 244 489 505 531

UGT1A6

A. PCR

F(-53) → 19T>G / F414 → 541A>G / 552A>C

Exon 1A6

← R184   ← R628

B. Digest

Product 1
237 bp
HhaI

T

G

Product 2
215 bp
NsiI

A

G

BbvI

A

C

C.

19	
T	G

HhaI

237 bp —
165 bp —
72 bp —

541	
A	G

NsiI

215 bp —
128 bp —
87 bp —

552		
A	C	A/C

BbvI

215 bp —
139 bp —
76 bp —

α-Naphthol

Mean Rate of Glucuronidation (nmol/min/mg)

Microsome Genotype

*1/*1 *1/*2 *1/*3 *1/*4 *2/*2 *2/*3

p < 0.05
p < 0.001

p-Nitrophenol

Mean Rate of Glucuronidation (nmol/min/mg)

Microsome Genotype

*1/*1 *1/*2 *1/*3 *1/*4 *2/*2 *2/*3

p < 0.05
p < 0.001

A.

rUGT1A6 (Units)

0.64 0.32 0.16

Representative Microsome Samples

053T 978T 380N 380T 455T 204T

49.5 kDa

B.

Immunoreactive UGT1A6 (Relative Units)

UGT1A6 Genotypes

*1/*1 *1/*2 *1/*3 *1/*4 *2/*2 *2/*3

C.

α-Naphthol

Normalized glucuronidation rate (nmol/min/mg/Unit UGT1A6)

p-Nitrophenol

Normalized glucuronidation rate (nmol/min/mg/Unit UGT1A6)

Microsome Genotype

A.

NsiI

*2/*2   *2/*1   *1/*1   pcDNA3.1   100 bp ladder

Size (bp)

300

200

100

B.

Legend:
- ■ *1/*1
- ✱ *1/*2
- □ *2/*2

X-axis: p-nitrophenol (mM)

Y-axis: v (nmol/min/mg)